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Abstract

Among the nine *Echinacea* species, *E. purpurea*, *E. angustifolia* and *E. pallida*, have been widely used to treat the common cold, flu and other infections. In this study, ethanol extracts of these three *Echinacea* species and *E. paradoxa*, including its typical variety, *E. paradoxa* var. *paradoxa*, were screened in lipopolysaccharide (LPS)-stimulated macrophage cells to assess potential anti-inflammatory activity. *E. paradoxa* var. *paradoxa*, rich in polyenes/polyacetylenes, was an especially efficient inhibitor of LPS-induced production of nitric oxide (NO), prostaglandin E2 (PGE2), interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6) by 46%, 32%, 53% and 26%, respectively, when tested at 20 μ g/ml in comparison to DMSO control. By bioactivity-guided fractionation, pentadeca-8Z-ene-11, 13-diyn-2-one (Bauer ketone 23) and pentadeca-8Z, 13Z-dien-11-yn-2-one (Bauer ketone 24) from *E. paradoxa* var. *paradoxa* were found primarily responsible for inhibitory effects on NO and PGE2 production. Moreover, Bauer ketone 24 was the major contributor to inhibition of inflammatory cytokine production in LPS-induced mouse macrophage cells. These results provide a rationale for exploring the medicinal effects of the Bauer ketone-rich taxon, *E. paradoxa* var. *paradoxa*, and confirm the anti-inflammatory properties of Bauer ketones 23 and 24.

Keywords

Echinacea, Ethanol extract, Fractionation, Polyacetylenes, Ketoalkenynes, Polyenes, Ketoalkenes, Anti-inflammatory

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Comments

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Bauer ketones 23 and 24 from *Echinacea paradoxa* var. *paradoxa* inhibit lipopolysaccharide-induced nitric oxide, prostaglandin E2 and cytokines in RAW264.7 mouse macrophages

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Among the nine *Echinacea* species, *E. purpurea*, *E. angustifolia* and *E. pallida*, have been widely used to treat the common cold, flu and other infections. In this study, ethanol extracts of these three *Echinacea* species and *E. paradoxa*, including its typical variety, *E. paradoxa* var. *paradoxa*, were screened in lipopolysaccharide (LPS)-stimulated macrophage cells to assess potential anti-inflammatory activity. *E. paradoxa* var. *paradoxa*, rich in polyenes/polyacetylenes, was an especially efficient inhibitor of LPS-induced production of nitric oxide (NO), prostaglandin E2 (PGE2), interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6) by 46%, 32%, 53% and 26%, respectively, when tested at 20 μ g/ml in comparison to DMSO control. By bioactivity-guided fractionation, pentadeca-8Z-ene-11, 13-diyn-2-one (Bauer ketone 23) and pentadeca-8Z, 13Z-dien-11-yn-2-one (Bauer ketone 24) from *E. paradoxa* var. *paradoxa* were found primarily responsible for inhibitory effects on NO and PGE2 production. Moreover, Bauer ketone 24 was the major contributor to inhibition of inflammatory cytokine production in LPS-induced mouse macrophage cells. These results provide a rationale for exploring the medicinal effects of the Bauer ketone-rich taxon, *E. paradoxa* var. *paradoxa*, and confirm the anti-inflammatory properties of Bauer ketones 23 and 24.

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1. Introduction

Echinacea preparations are among the most widely used dietary supplements and their annual sales gross \$120 million in the US (2009). There are two main taxonomic systems for *Echinacea*: McGregor's taxonomy (nine species and four varieties, used herein) (McGregor, 1968) and a more recent morphometric analysis-based classification (four species and eight varieties) (Binns et al., 2002b). Three species, *Echinacea angustifolia*, *Echinacea purpurea* and *Echinacea pallida*, are commonly used for the prevention and treatment

of upper respiratory tract infections, such as the common cold and influenza, in North America and Europe.

The efficacy of *Echinacea* for the treatment of the common cold, such as shortening duration and alleviating symptoms, has been observed in some clinical trials, but no clear evidence has demonstrated prevention of colds (Linde et al., 2006). However, the heterogeneity of clinical studies, in experimental design, *Echinacea* preparations used, and outcomes measured, has led to conflicting results (Linde et al., 2006). Moreover, the lack of information regarding the chemical composition of *Echinacea* preparations in many past published studies has made their outcomes difficult to interpret and compare.

There is a large body of evidence, based on cell culture and animal studies, demonstrating that *Echinacea* extracts possess immunomodulatory, anti-inflammatory, antiviral, antioxidant and antimicrobial properties (Barnes et al., 2005). It has been reported that polysaccharides, cichoric acid and alkamides might contribute to immunological activity via enhancing cytokine production and

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phagocytic activity of macrophages (Goel et al., 2002; Stimpel et al., 1984). The antiviral effects of *Echinacea* have been attributed to glycoproteins and cichoric acid (Barnes et al., 2005; Bodinet and Buescher, 1991). Anti-inflammatory activity has been observed with a polysaccharide fraction and with polyunsaturated alkamides from *E. angustifolia* roots, and echinacoside, alkamides and polyenes/polyacetylenes from *E. pallida* (LaLone et al., 2007, 2009; Tubaro et al., 1987). In addition, polyenes and polyacetylenes from *E. pallida* roots have also been reported to induce apoptosis of tumor cells (Chicca et al., 2008).

In this research, the anti-inflammatory activity of *Echinacea* species was investigated by using LPS-stimulated RAW264.7 mouse macrophage cells as the research model. PGE2, NO and inflammatory cytokines, secreted by RAW264.7 macrophages under stimulation with LPS, are critical endpoints to evaluate the activation of macrophages and the magnitude of inflammatory responses. Two goals were addressed: (1) to compare the effectiveness of ethanol extracts from the roots of various *Echinacea* species on the production of PGE2, NO and inflammatory cytokines from LPS-stimulated RAW264.7 macrophages; and (2) to identify the constituents responsible for any observed bioactivity of these *Echinacea* ethanol extracts, and assess the effects of identified compounds on expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), the key enzymes to regulate production of PGE2 and NO, respectively. All compounds mentioned throughout the text were numbered and shown in Fig. 1.

2. Results and discussion

2.1. Inhibition of NO, PGE2 and inflammatory cytokines production by *Echinacea* species/accessions

To assess the anti-inflammatory effects of selected *Echinacea* species and accessions, six accessions from four *Echinacea* species (Table 1) were screened for their ability to inhibit LPS-induced inflammatory response in RAW264.7 macrophages. For all endpoints (NO, PGE2, IL-6, IL-1 β and TNF- α), the extracts were tested at a normalized concentration of 20 μ g/ml to compare anti-inflammatory activity. All the treatments with *Echinacea* extracts were compared to the DMSO vehicle control treatment with or without LPS induction in RAW264.7 cells. Cytotoxicity screening showed no cytotoxicity with any of these extracts at the screened concentrations (data not shown).

As shown in Table 2, all six *Echinacea* ethanol extracts significantly reduced NO production in LPS-stimulated RAW264.7 cells. Two accessions of *E. paradoxa* showed the highest inhibitory activity with reduction of LPS-induced NO levels by 39% and 46% when compared to their corresponding controls. LPS-induced PGE2 levels were significantly inhibited by ethanol extracts from *E. purpurea*, *E. pallida* Ames 28968, and *E. paradoxa* var. *paradoxa*. LPS-induced IL-1 β and IL-6 levels were inhibited by *E. paradoxa* var. *paradoxa* ethanol extract most potently and by *E. pallida* PI 631274 to a lesser extent. A slight decline in TNF- α production was observed in LPS-induced RAW264.7 cells treated with *E. purpurea*, *E. paradoxa* and both accessions of *E. pallida*. In the absence of LPS, all *Echinacea* ethanol extract treatments of RAW264.7 cells stimulated TNF- α level, but levels of IL-1 β and IL-6 production were undetectable (data not shown). It has also been observed that *Echinacea purpurea* ethanol extract stimulated cytokine production in uninfected epithelial cells but inhibited cytokine production in rhinovirus-infected epithelial cells (Sharma et al., 2006). This reveals the complexity of *Echinacea* constituents: selected compounds might exhibit immunostimulatory properties, which could be overwhelmed by the anti-inflammatory activity of some other compounds in the course of LPS or virus infection. As expected,

quercetin (compound 3) significantly inhibited LPS-stimulated NO, PGE2, IL-1 β and IL-6 levels at 10 μ M, which was consistently observed among all the experiments.

2.2. Inhibition of NO, PGE2 and inflammatory cytokines production by fractions from *E. paradoxa* var. *paradoxa* extracts

Considering the very potent inhibition of LPS-induced NO, PGE2, IL-1 β and IL-6 by the ethanol extract of *E. paradoxa* var. *paradoxa*, the ethanol extract of accession PI 631292 by was then fractionated semi-preparative HPLC (Table 3) and screened individual fractions for anti-inflammatory activity. The concentrations of fractions 1–6 screened for cytotoxicity on RAW264.7 cells were tested at the highest concentrations obtained from fractionation (Table 4). Fraction 3 at 101 μ g/ml and fraction 6 at 187 μ g/ml significantly decreased cell viability. No cytotoxicity was detected with fractions 3 and 6 at 50 μ g/ml or for any other fractions at the highest concentrations tested.

Fractions 3, 4, 5 and 6 at the highest concentrations tested dramatically reduced NO and PGE2 production (Fig. 2A). The observed cytotoxicity of fractions 3 and 6 at the concentrations studied may have partially contributed to reduction of NO and PGE2.

The activities of fractions 3–6 were compared at non-toxic doses (Fig. 2B). By comparing the effects on NO and PGE2 production caused by these four fractions at 3.2 μ g/ml, fractions 4 and 5 had the greatest inhibitory ability on PGE2, and fraction 4 most potently inhibited NO. Fraction 5 inhibited NO and PGE2 levels to a greater extent than did fractions 3 or 6 when all were tested at 20 μ g/ml. The inhibition of PGE2 levels by fractions 3, 5 and 6 and the inhibition of NO levels by fractions 3 and 5 all showed a dose-dependent pattern.

The effects of fractions 1–6 on LPS-induced inflammatory cytokines were also examined. LPS-stimulated IL-1 β and IL-6 levels were significantly reduced by fractions 3, 4, 5 and 6 at their highest concentrations (Table 5). When the tested concentrations of fractions 3, 5 and 6 were normalized to the non-cytotoxic dose of 20 μ g/ml, fraction 5 showed the greatest inhibition of IL-1 β and IL-6 induction. LPS induction of TNF- α was significantly alleviated by fractions 4, 5 and 6 at highest doses, but not at 20 μ g/ml. When RAW264.7 cells were treated with fractions 1–6 at the highest concentrations without LPS stimulation, different trends were observed (Table 5). Fractions 3 and 5 reduced baseline TNF- α production by 44% and 36%, respectively, whereas fraction 6 induced TNF- α levels 2.4 \times above those observed with the DMSO control. When the fractions were diluted to 20 μ g/ml, only fraction 5 inhibited TNF- α levels (by 24%).

Although fraction 4 at 3.2 μ g/ml and fraction 5 at 36 μ g/ml similarly suppressed the production of inflammatory mediators, fraction 5 contributed more to extract activity, since the concentration of fraction 5 proportional to yield (144 μ g/ml) was much higher than the amount tested (36 μ g/ml) and fraction 5 exhibited a dose-dependent activity.

2.3. Effects of combined fractions 4 and 5 of *E. paradoxa* var. *paradoxa* on NO and PGE2 inhibition

Since the most potent anti-inflammatory activities were observed with fractions 4 and 5, these were combined examine their joint effect on LPS-induced NO and PGE2 production. When fractions 4 and 5 at the highest concentrations were applied in the RAW264.7 cells with LPS stimulation alone and together, the combined fractions demonstrated synergistic inhibition of NO production, as indicated in Fig. 3A. In parallel studies of LPS-induced PGE2, it was necessary to dilute fraction 5 to 3.2 μ g/ml because fraction 5 alone reduced LPS induced PGE2 by 96% (\pm 1%). Fraction 4 at 1.6 μ g/ml and fraction 5 at 3.2 μ g/ml each resulted in a moderate reduction

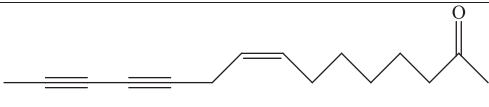
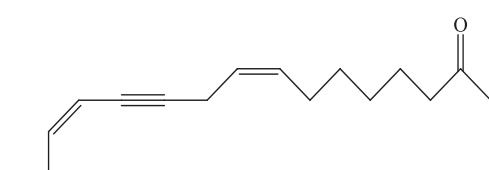
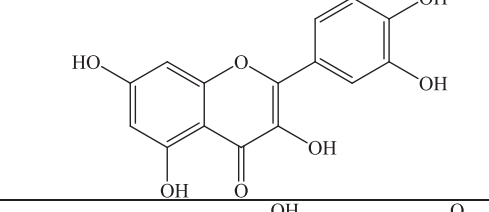
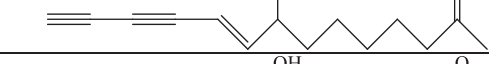
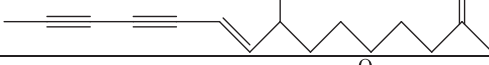

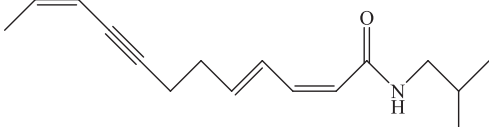
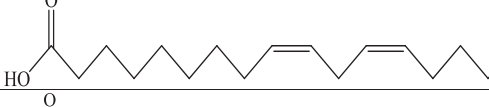

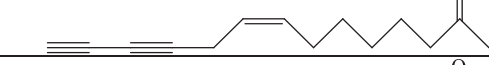
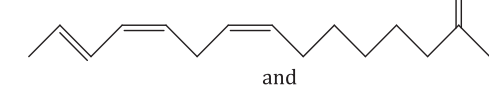
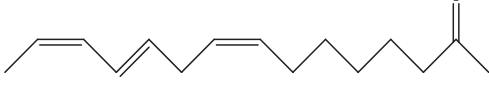
Compound 1	Bauer ketone 23	
Compound 2	Bauer ketone 24	
Compound 3	Quercetin	
Compound 4	Bauer ketone 20	
Compound 5	Bauer ketone 21	
Compound 6	Alkamide 2	
Compound 7	Alkamide Chen 1	
Compound 8	Linoleic acid	
Compound 9	α -linolenic acid	
Compound 10	Bauer ketone 22	
Compound 11	Bauer ketone 25	
		and 

Fig. 1. Compounds 1–11.

of PGE₂, and, when combined, these fractions resulted in greater inhibition of PGE₂ (Fig. 3B).

The effects of combined fractions 4 (1.6 μ g/ml) and 5 (3.2 μ g/ml) on LPS induced IL-1 β , IL-6 and TNF- α levels at 8 h were also determined (data not shown). Combined fractions significantly inhibited IL-6 to a greater extent than did the individual fractions, showing a synergistic effect. However, LPS-stimulated TNF- α level was not altered by fraction 4 or/and fraction 5 at 8 h. IL-1 β was not elevated in LPS treated macrophages at 8 h.

2.4. GC–MS analysis of active fractions of *E. paradoxa* var. *paradoxa* ethanol extract

GC–MS was employed to identify the constituents in the active fractions 3–6. As indicated in Fig. 4, Bauer ketones 20 (compound 4) and 21 (compound 5) were identified in fraction 3. Three classes of compounds were identified in fraction 4: (1) polyacetylenes and polyenes, including alkamide 2 (compound 6), alkamide Chen 1 (compound 7) and Bauer ketone 21 (compound 5); (2) polyunsat-

Table 1Provenances and vouchers of *Echinacea* accessions evaluated.^a

Taxon	Accession No.	Provenance	Herbarium voucher
<i>E. angustifolia</i>	Ames 24996	Burleigh Co., North Dakota	M.P. Widrechner, 26 June 2009
<i>E. pallida</i>	Ames 28968	Synthetic populations from Central Iowa	M.P. Widrechner, 2 July 2009
<i>E. pallida</i>	PI 631274	Creek Co., Oklahoma	M.P. Widrechner, 2 July 2009
<i>E. paradoxa</i>	Ames 27724	Laclede Co., Missouri	J. McCoy, 19 October 2004
<i>E. paradoxa</i> var. <i>paradoxa</i>	PI 631292	Stone Co., Arkansas	M.P. Widrechner, 2 July 2009
<i>E. purpurea</i>	Ames 27468	Madison Co., Ohio	M.P. Widrechner, 26 June 2009

^a All vouchers are deposited at ISC (Ada Hayden Herbarium, Iowa State University, Ames, Iowa).**Table 2**Effects of *Echinacea* ethanol extracts on production of inflammatory mediators in RAW264.7 cells.

Treatments ^a	Endpoints ^b					
	NO (% of DMSO + LPS control) ^c	PGE2 (% of DMSO + LPS control) ^c	IL-1 β (% of DMSO + LPS control) ^c	IL-6 (% of DMSO + LPS control) ^c	TNF- α (% of DMSO + LPS control) ^c	TNF- α (% of DMSO) ^d
<i>E. angustifolia</i> (Ames 24996)	103 \pm 5	77 \pm 4	90 \pm 4	104 \pm 3	101 \pm 4	195 \pm 23
<i>E. pallida</i> (Ames 28968)	69 \pm 8	80 \pm 1	82 \pm 3	83 \pm 3	87 \pm 4	171 \pm 16
<i>E. pallida</i> (PI 631274)	77 \pm 2	62 \pm 2	72 \pm 2	81 \pm 2	88 \pm 4	143 \pm 12
<i>E. paradoxa</i> (Ames 27724)	61 \pm 1	87 \pm 6	80 \pm 8	80 \pm 2	89 \pm 1	227 \pm 44
<i>E. paradoxa</i> var. <i>paradoxa</i> (PI 631292)	54 \pm 5	68 \pm 2	47 \pm 3	74 \pm 5	95 \pm 3	199 \pm 24
<i>E. purpurea</i> (Ames 27468)	77 \pm 4	68 \pm 4	87 \pm 2	92 \pm 3	93 \pm 3	352 \pm 33
Quercetin (compound 3)	37 \pm 3	22 \pm 9	26 \pm 5	66 \pm 5	103 \pm 2	72 \pm 3

^a RAW264.7 cells were treated with six *Echinacea* ethanol extracts at 20 μ g/ml, DMSO control and 10 μ M of quercetin, respectively, with or without 1 μ g/ml LPS. Quercetin was included as a positive control, inhibiting LPS-induced NO and PGE2 production significantly.^b The levels of inflammatory mediators were detected after 8 h (PGE2) or 24 h (NO, IL-1 β , IL-6 and TNF- α) treatment.^c The (Media + DMSO + LPS) control group was standardized at 100% of production of NO (10.9 \pm 0.7 μ M), PGE2 (3.38 \pm 0.20 ng/ml), IL-1 β (140.5 \pm 14.3 pg/ml), IL-6 (23.1 \pm 1.0 ng/ml) and TNF- α (30.8 \pm 1.2 ng/ml). Data were expressed as % of the (Media + DMSO + LPS) control \pm SE (N = 3). Bold and italics mean significant difference (bold $p \leq 0.05$ and italics $p \leq 0.001$) from the (Media + DMSO + LPS) control group.^d The (Media + DMSO) control group was normalized to 100% of TNF- α production (0.18 \pm 0.02 ng/ml). Data were expressed as % of the (Media + DMSO) control \pm SE (N = 3). Treatments without LPS revealed no change in the other endpoints compared to (Media + DMSO) controls (data not shown).**Table 3**(A) Solvent gradient for fractionation of *E. paradoxa* var. *paradoxa* ethanol extract. (B) Elution time periods for fraction collection.

(A)								
Time (min)	0–13	13–14	14–21	21–66	66–69	69–73	73–75	75–80
Solvent A ^a (%)	90–80	80–60	60	60–20	20–0	0	0–90	90
Solvent B ^b (%)	10–20	20–40	40	40–80	80–100	100	100–10	10
(B)								
Fractions	1	2	3	4	5	6		
Elution time (min)	5–11	11–29	29–51	51–60	60–67	67–80		
Concentration (mg/ml)	184	75	101	3.2	36	187		

^a Solvent A: 0.1% acetic acid in endotoxin-free water.^b Solvent B: acetonitrile.**Table 4**Cytotoxicity of six fractions from *Echinacea paradoxa* var. *paradoxa*.^a

Fractions	Yield (mg)	Concentration ^b (μ g/ml)	Viability (% of DMSO control \pm SE, p -value)	Diluted concentration (μ g/ml)	Viability (% of DMSO control \pm SE, p -value)
1	920	184	93 \pm 7 (0.262)	–	–
2	93	75	90 \pm 3 (0.1338)	–	–
3	25.2	101	68 \pm 3 (<0.0001)	50	108 \pm 9 (0.3911)
4	0.8	3.2	89 \pm 8 (0.0764)	–	–
5	36	36	104 \pm 3 (0.5964)	–	–
6	70	187	55 \pm 3 (<0.0001)	50	121 \pm 3 (0.0046)

^a The viability of each fraction was denoted as % of DMSO control \pm SE (N = 3). The number in brackets represents the p -value for comparison of fraction treatment groups to the DMSO control group. Boldface p -value refers to significant difference ($p < 0.05$).^b The tested concentrations of each fraction were the highest concentrations obtained from the fractionation. They were also used in the NO, PGE2 and inflammatory cytokine screening (Fig. 1A and Table 5).

urated fatty acids, including linoleic acid (compound **8**) and α -linolenic acid (compound **9**); and (3) carbohydrates. Fraction 5 was rich in Bauer ketones 22 (compound **10**), 23 (compound **1**) and 24 (compound **2**), which comprised 96% of total dry weight Bauer ketones 23 (compound **1**) and 24 (compound **2**) were also found in fraction 6. There were also unidentified peaks in each fraction.

Alkamide 2 and Chen alkamide 1 (compounds **6**, **7**) identified in fraction 4 have been reported to inhibit PGE2 production significantly at 50 μ M (LaLone et al., 2007). However, alkamide 2 or Chen alkamide 1 (compounds **6**, **7**) could not account for the activity of fraction 4, since neither of them reached 20 μ M in fraction 4 at 3.2 μ g/ml. Two essential unsaturated fatty acids, linoleic acid and

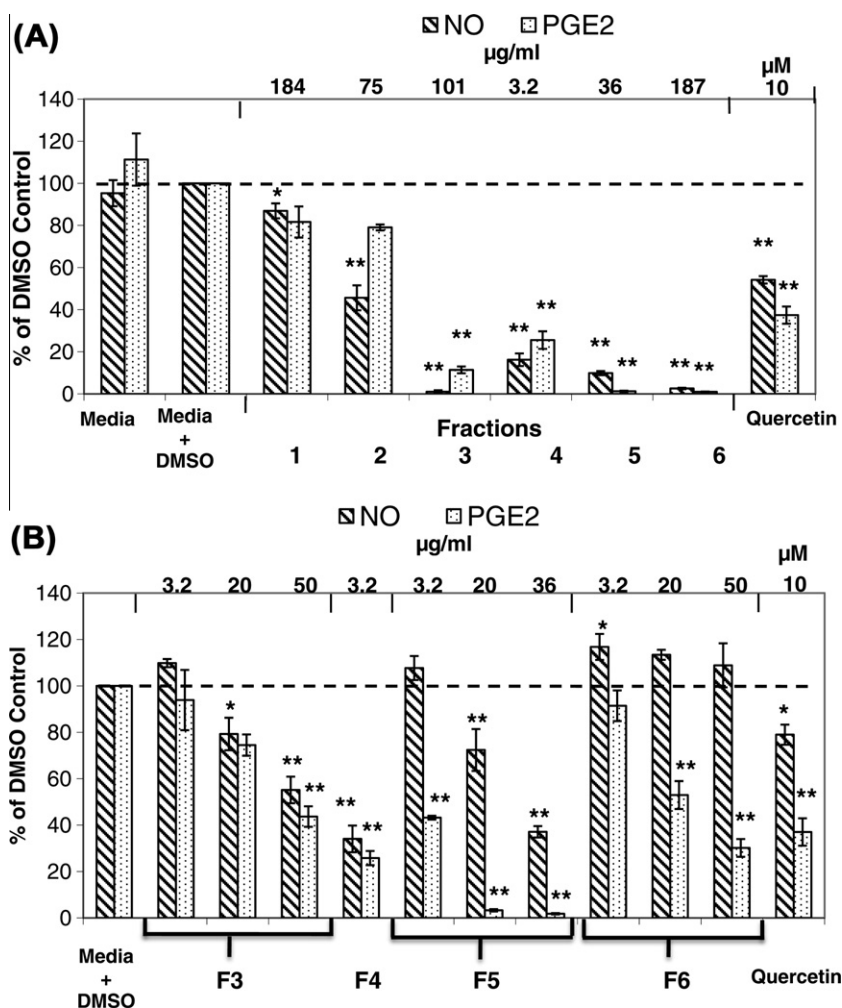


Fig. 2. Inhibition of NO and PGE2 production by the fractions from a 2009 extract of *Echinacea paradoxa* var. *paradoxa* (PI 631292) in LPS stimulated RAW264.7 cells. (A) Production of NO and PGE2 after treatment with six fractions at their highest concentrations obtained. (B) Production of NO and PGE2 after treatment of fractions 3, 5 and 6 at three doses up to the highest dose obtained or the dose shown to be non-toxic (Table 3). The data were expressed as % of (Media + DMSO + LPS) control \pm SE ($N = 3$), and the (Media + DMSO + LPS) control group was set at 100% of NO production ($15.5 \mu\text{M} \pm 0.4$ in (A) and $18.9 \pm 1.7 \mu\text{M}$ in (B)) and PGE2 production ($2.8 \pm 0.2 \text{ ng/ml}$ in (A) and $3.3 \pm 0.1 \text{ ng/ml}$ in (B)). Asterisks reflect significant differences (* $p < 0.05$ and ** $p < 0.001$) from the (Media + DMSO + LPS) control group. Treatments without LPS revealed no change compared to (Media + DMSO) controls (data not shown).

Table 5
Effects of fractions from *Echinacea paradoxa* var. *paradoxa* on inflammatory cytokine production in RAW264.7 Cells.

Treatments		Endpoints			
Fractions	Concentration ($\mu\text{g/ml}$)	IL-1 β (% of DMSO + LPS control) ^a	IL-6 (% of DMSO + LPS control) ^a	TNF- α (% of DMSO + LPS control) ^a	TNF- α (% of DMSO) ^b
1	184	89 \pm 7	88 \pm 3	94 \pm 1	110 \pm 6
2	75	73 \pm 7	86 \pm 3	99 \pm 3	104 \pm 2
3	101	31 \pm 2	39 \pm 4	90 \pm 1	56 \pm 4
	20	59 \pm 4	89 \pm 1	97 \pm 4	98 \pm 9
4	3.2	57 \pm 3	51 \pm 2	85 \pm 4	81 \pm 7
5	36	11 \pm 2	42 \pm 1	78 \pm 4	64 \pm 8
	20	21 \pm 3	59 \pm 3	89 \pm 1	76 \pm 5
6	187	2 \pm 1	50 \pm 3	43 \pm 6	236 \pm 10
	20	55 \pm 3	74 \pm 4	95 \pm 4	87 \pm 12
Quercetin (Compound 3)	10 μM	10 \pm 2	53 \pm 1	107 \pm 7	81 \pm 17

^a The levels of IL-1 β , IL-6 and TNF- α after treatment with *Echinacea paradoxa* var. *paradoxa* fractions at the highest concentrations and 20 $\mu\text{g/ml}$ for fractions 3, 5 and 6 were compared to those of the (Media + DMSO + LPS) control group in 1 $\mu\text{g/ml}$ LPS induced RAW264.7 cells. Data were expressed as mean \pm SE ($N = 3$). IL-1 β production, IL-6 production and TNF- α production of the (Media + DMSO + LPS) control group were $143.6 \pm 14.5 \text{ pg/ml}$, $27.1 \pm 0.9 \text{ ng/ml}$ and $39.8 \pm 2.3 \text{ ng/ml}$, respectively. Bold and italics represent significant differences (bold $p < 0.05$ and italics $p < 0.001$) from the (Media + DMSO + LPS) control group.

^b The levels of TNF- α with the above-mentioned treatment groups were compared to that of the (Media + DMSO) control group ($0.41 \pm 0.02 \text{ ng/ml}$) in RAW264.7 cells without LPS addition. Treatments without LPS revealed no change in the other endpoints compared to (Media + DMSO) controls (data not shown).

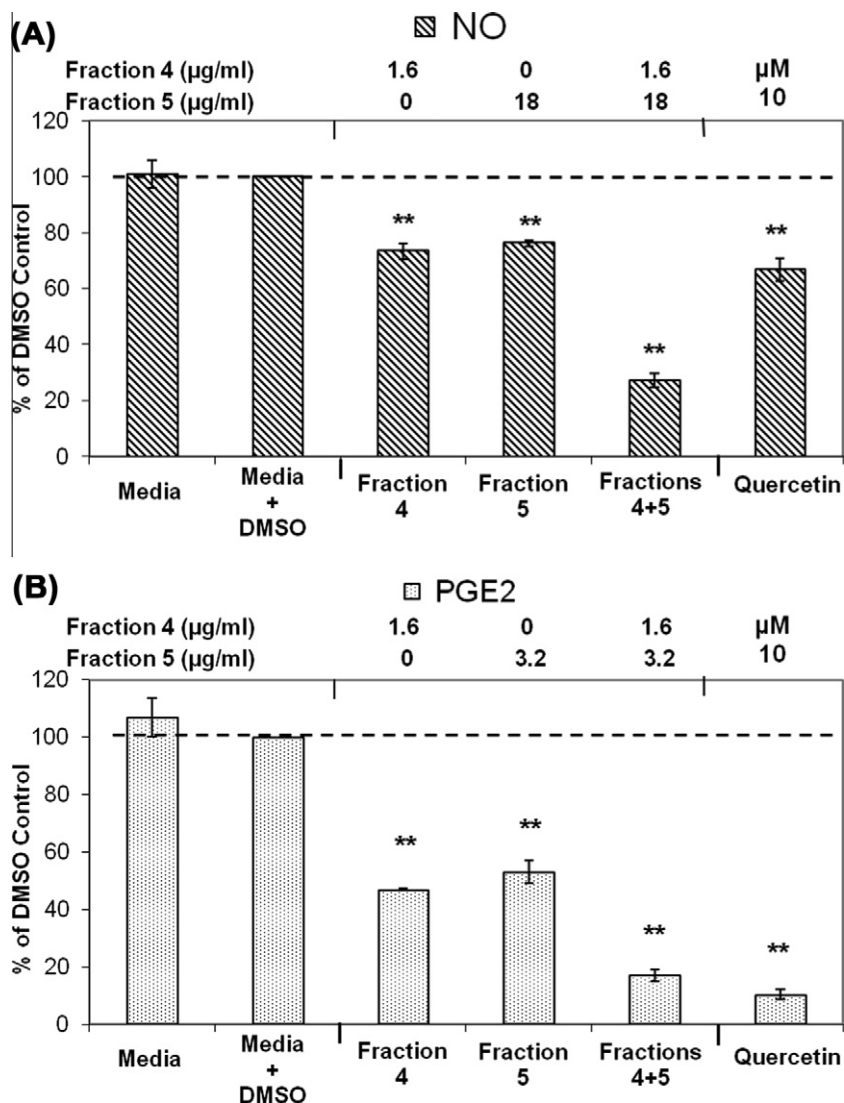


Fig. 3. Synergistic inhibition of NO (A) and additive inhibition of PGE2 (B) production by fractions 4 and 5 from *Echinacea paradoxa* var. *paradoxa* in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with combined or individual fraction 4 and fraction 5 at indicated concentrations, with 1 μg/ml LPS. Data were expressed as % of the (Media + DMSO + LPS) control \pm SE ($N=3$), and the (Media + DMSO + LPS) control group was set at 100% of NO production (14.8 ± 0.7 μM) and PGE2 (2.2 ± 0.4 ng/ml) production. Asterisks reflect significant differences (** $p < 0.001$) from the (Media + DMSO + LPS) treatment group. Treatments without LPS revealed no change compared to (Media + DMSO) controls (data not shown).

α -linolenic acid (ALA) (compounds **8**, **9**), were also identified in the fraction 4. ALA (compound **9**), an ω -3 PUFA found mainly in plants, was reported to have anti-inflammatory activity in the animal model (carrageenan-induced paw edema in rats) (Ren et al., 2007). In the RAW264.7 cell model, ALA (compound **9**) was found to inhibit LPS-induced NO and PGE2 production, and protein and mRNA expression levels of the iNOS and COX-2 enzymes (Ren et al., 2007). However, further fractionation of fraction 4 did not yield any potent subfraction, indicating that constituent interactions might be responsible for the majority of its activity.

2.5. Dose-dependent activity of Bauer ketones identified in fraction 5 of *E. paradoxa* var. *paradoxa*

Due to its promising anti-inflammatory activities and relatively simple chemical profile, the components in fraction 5 were further quantified by GC-MS (Table 6). Fraction 5 (3.2 μg/ml) by dry weight was composed of 3.1 μM Bauer ketone 22 (compound **10**), 1.6 μM Bauer ketone 23 (compound **1**) and 9.7 μM Bauer ketone 24 (compound **2**).

To investigate the bioactivity of Bauer ketones, three doses of Bauer ketones 22, 23 and 24 (compounds **10**, **1**, **2**) were applied in RAW264.7 cells with or without LPS induction, as described in Section 4. As indicated in Fig. 5A, all three doses of Bauer ketone 24 (compound **2**) significantly decreased LPS-induced NO production with a dose-dependent relationship. Bauer ketone 23 (compound **1**) significantly inhibited NO induction at 10 and 5 μM, and Bauer ketone 22 (compound **10**) resulted in a moderate decrease of NO level at 10 μM. Dose-dependent inhibition of LPS-induced PGE2 production was observed with Bauer ketones 22, 23 and 24 (compounds **10**, **1**, **2**) (Fig. 5A). All three doses of Bauer ketone 23 (compound **1**) significantly reduced PGE2 levels, but Bauer ketones 22 and 24 (compounds **10**, **2**) did not affect PGE2 levels significantly at 1.6 μM.

2.6. Contribution of Bauer ketones in fraction 5 of *E. paradoxa* var. *paradoxa* to inhibition of NO, PGE2 and inflammatory cytokine production

To determine the degree to which Bauer ketones 22, 23 and 24 (compounds **10**, **1**, **2**) at concentrations found in fraction 5 contrib-

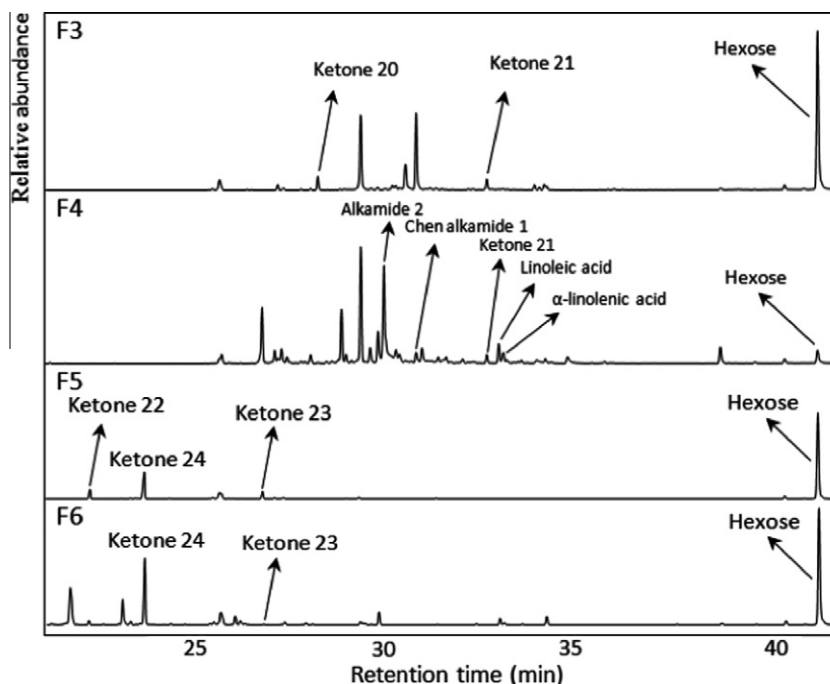


Fig. 4. GC-MS analysis of bioactive fractions from *E. paradoxa* var. *paradoxa*. Total ion chromatograms of fractions 3, 4, 5 and 6 are displayed. Peaks were identified by comparing their retention time and mass spectra to authentic standards.

uted to the anti-inflammatory activity observed when that fraction was tested at 3.2 $\mu\text{g/ml}$, the effects of individual or combined synthesized Bauer ketones on LPS-stimulated NO, PGE2 and pro-inflammatory cytokine production was examined. As shown in Fig. 5B, when compared to (Media + DMSO + LPS) control, all treatments with Bauer ketone 24 (compound **2**), including Bauer ketones 22 + 23 + 24 (compounds **10** + **1** + **2**), Bauer ketones 22 + 24 (compounds **10** + **2**), Bauer ketones 23 + 24 (compounds **1** + **2**), and Bauer ketone 24 (compound **2**) alone, decreased LPS-induced NO production significantly. Bauer ketone 22 (compound **10**) did not exert any effect on NO levels. Multiple comparison with Tukey adjustment was employed to compare among the effects we observed from combined and individual Bauer ketones and from fraction 5 at 3.2 $\mu\text{g/ml}$. Despite the lack of an effect by Bauer ketone 23 (compound **1**) alone, Bauer ketones 23 + 24 (compounds **1** + **2**) did inhibit NO production to a greater extent than did Bauer ketone 24 (compound **2**) alone, which suggested that the interaction of Bauer ketones 23 and 24 (compounds **1**, **2**) could result in stronger inhibition of LPS-induced NO production. A similar relationship was found between Bauer ketones 22 + 23 + 24 (compounds **10** + **1** + **2**) and Bauer ketones 22 + 24 (compounds **10** + **2**). Although Bauer ketones have inhibitory properties at concentrations present in fraction 5 at 3.2 $\mu\text{g/ml}$, fraction 5 itself did not exhibit any effect on NO induction at 3.2 $\mu\text{g/ml}$. This suggests that minor constituents might counteract Bauer ketones, thus weakening the anti-inflammatory potential of fraction 5. Moreover, fraction 5 at 20 $\mu\text{g/ml}$ and Bauer ketones 23 + 24 (compounds **1** + **2**) reduced LPS-stimulated NO production to the same extent.

LPS-stimulated PGE2 production was dramatically reduced by all treatments with Bauer ketones ($p < 0.001$), except for the treatment with Bauer ketone 22 (compound **10**) alone. Multiple comparison demonstrated that reduction of PGE2 by Bauer ketones 23 + 24 (compounds **1** + **2**) was significantly greater than that by individual Bauer ketones 23 (compound **1**) or 24 (compound **2**). Addition of Bauer ketone 22 (compound **10**) did not affect the degree of inhibition caused by Bauer ketones 23 or/and 24 (compounds **1**, **2**). No significant difference was found between reduction of PGE2 by fraction

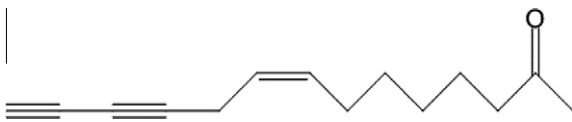
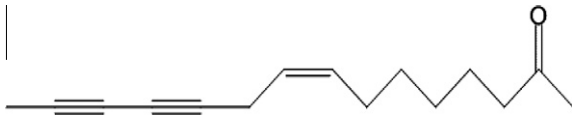
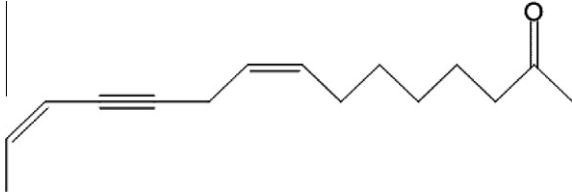
5 and that by Bauer ketones 23 and/or 24 (compounds **1**, **2**). This indicated that Bauer ketones 23 and 24 (compounds **1**, **2**) contributed to PGE2 inhibition by fraction 5 at 3.2 $\mu\text{g/ml}$.

Synthesized Bauer ketones also exerted inhibitory effects on LPS-induced IL-1 β and IL-6 production. As shown in Fig. 6A, all the treatments containing Bauer ketone 24 (compound **2**) dramatically inhibited IL-1 β induction compared to (Media + DMSO + LPS) control. Fig. 6B illustrates a moderate decrease of IL-6 induction by all treatments containing Bauer ketone 24 (compound **2**) compared to (Media + DMSO + LPS) control. LPS-induced TNF- α production level was not changed by any treatments with Bauer ketones or fraction 5 (Fig. 6C). No cytotoxicity was found with Bauer ketones at the screened concentrations.

In summary, our results indicate that a combination of Bauer ketones 23 and 24 (compounds **1**, **2**) had the greatest potential to inhibit NO and PGE2 production, and Bauer ketone 24 (compound **2**) accounted for the inhibition of IL-1 β and IL-6 by fraction 5. Bauer ketone 22 (compound **10**) at 3.1 μM did not exert any effects on production of the tested endpoints. Some other minor constituents might have antagonistic effects which comprised the activity of Bauer ketones 23 and 24 (compounds **1**, **2**) as demonstrated by NO and IL-1 β screening. Results of testing three doses of Bauer ketones suggested that Bauer ketone 24 (compound **2**) was more effective in suppressing NO production and Bauer ketone 23 (compound **1**) was more effective for PGE2 inhibition. Although three Bauer ketones all exhibited anti-inflammatory potential to different extent, only Bauer ketones 23 and 24 (compounds **1**, **2**) contributed to the activity of fraction 5 considering their concentrations in the fraction 5 at 3.2 $\mu\text{g/ml}$. Combination of Bauer ketones at different ratios might lead to different level of inhibition.

Binns et al. (2002a) indicated that Bauer ketones were important chemotaxonomic markers based on the lipophilic phytochemical profiles of various *Echinacea* species. *E. paradoxa* and *E. pallida* exhibited similar pattern of ketoalkenynes, including Bauer ketones 22, 23, 24 and 25 (compounds **10**, **1**, **2**, **11**), but contained alkamides at low concentrations (Bauer and Foster, 1991; Bauer et al., 1988). In contrast, the roots of *E. purpurea* and *E. angustifolia*

Table 6
Quantitation of Bauer ketones in fraction 5.^a

Nomenclature	Structure	Concentration (μM in 3.2 $\mu\text{g/ml}$ of F5)
Tetradeca-8Z-ene-11,13-diyn-2-one (Bauer ketone 22, compound 10)		3.1
Pentadeca-8Z-ene-11,13-diyn-2-one (Bauer ketone 23, compound 1)		1.6
Pentadeca-8Z,13Z-dien-11-yn-2-one (Bauer ketone 24, compound 2)		9.7

^a Compounds were identified according to retention times and mass spectra from GC–MS. Synthesized Bauer ketones 22–24 were used as internal standards to quantify compounds in fraction 5.

were mainly characterized by the presence of alkamides (Bauer and Remiger, 1989).

Limited research has been conducted on the bioactivity of Bauer ketones. Our work demonstrates that *E. paradoxa* var. *paradoxa*, which is also rich in Bauer ketones, could partly attribute its anti-inflammatory activity (inhibitory ability on NO, PGE₂, IL-1 β and IL-6 production) to Bauer ketones 23 and 24 (compounds **1**, **2**). Prior results from our laboratory (LaLone et al., 2009, 2010) reported the inhibition of PGE₂ and NO production by these ketones at concentrations found in *E. pallida* and *E. angustifolia*, and the results in the present report with concentrations of Bauer ketones 23 and 24 (compounds **1**, **2**) that were found in *E. paradoxa* var. *paradoxa* were in agreement with the earlier results. Apart from its anti-inflammatory activity, Bauer ketone 24 (compound **2**) from *E. pallida* has also been reported to inhibit the growth of human cancer cell lines possibly by arresting the cell cycle in the G1 phase and promoting apoptotic cell death (Chicca et al., 2010). Bauer ketone 24 (compound **2**) is evidently permeable above $10 \times 10^{-6} \text{ cm s}^{-1}$ across the Caco-2 monolayer, suggesting potential oral bioavailability (Chicca et al., 2008). Therefore, Bauer ketone 24 (compound **2**) might be a promising candidate as an anti-inflammatory and anti-tumor agent. However, further investigation is necessary to confirm this in appropriate animal models.

2.7. Effects of Bauer ketones on the expression levels of COX-2 and iNOS

COX and iNOS are the critical enzymes catalyzing the committed step in the production of PGE₂ (Bakhle and Botting, 1996) and NO (MacMicking et al., 1997), respectively. COX-1 is constitutively expressed in various cells and tissues, but COX-2 is an inducible form in response to LPS or cytokines (Bakhle and Botting, 1996). To examine whether Bauer ketones 23 and 24 (compounds **1**, **2**) inhibit PGE₂ and NO levels through regulation of COX-2 and iNOS protein expression, RAW264.7 cells were treated with Bauer ketones at the concentrations present in 3.2 $\mu\text{g/ml}$ of fraction 5 to measure levels of COX-2 and iNOS by using Western blot, as described in Section 4.

As shown in Fig. 7 1 $\mu\text{g/ml}$ LPS induced the expression of COX-2 and iNOS, and COX-1 to a lesser extent. All the treatments did not alter the expression levels of COX-1 induced by LPS. Bauer ketone 24 (compound **2**) increased the expression of COX-2 significantly compared to DMSO control in LPS-induced macrophages, whereas

only a trend towards increased COX-2 was observed with Bauer ketone 23 (compound **1**). The combined Bauer ketones were more effective than were individual Bauer ketones, with a 2-fold increase in COX-2 levels in relation to (Media + DMSO + LPS) control. LaLone et al. (2010) reported that Bauer ketone 23 (compound **1**) identified in an *E. angustifolia* extract had the capacity to increase COX-2 expression significantly at 5 μM in LPS-stimulated macrophages. In contrast, Bauer ketone 23 (compound **1**) inhibited LPS-induced COX-2 activity at 0.83 μM (LaLone et al., 2010). Therefore, it is speculated that Bauer ketones 23 and 24 (compounds **1**, **2**) might reduce activity of COX-2 enzyme rather than decrease COX-2 expression at translational levels, and consequently inhibit PGE₂ production in LPS-stimulated macrophages. However, the mechanism underlying an increase in the amount of COX-2 protein is unclear.

Although inhibition of iNOS expression has been found with alkamides (Hou et al., 2010), neither Bauer ketones nor fraction 5 from *E. paradoxa* affected LPS-induced iNOS expression. This indicates that the inhibition of NO production by Bauer ketones 23 + 24 (compounds **1** + **2**) cannot be attributed to iNOS regulation at the protein level. α -Tubulin, as a loading control, was constitutively expressed among all treatments (data not shown).

3. Conclusions

Echinacea species, especially the extensively studied *E. purpurea*, have been found to inhibit inflammatory mediators in different cell model systems (Canlas et al., 2010; Sharma et al., 2010). Our experiments demonstrate that (1) *E. paradoxa* var. *paradoxa* (PI 631292) has more potent anti-inflammatory activity as measured by inflammatory mediators including NO, PGE₂, IL-1 β and IL-6 in RAW264.7 cells than do the three most common medicinal species of *Echinacea* (Table 4); (2) Bauer ketones from *E. paradoxa* var. *paradoxa* played an important role in the inhibition of inflammatory mediator production in LPS-stimulated RAW264.7 cells. Bauer ketones 23 and 24 (compounds **1**, **2**) were identified as key constituents contributing to NO and PGE₂ inhibition by *E. paradoxa* var. *paradoxa*. Moreover, Bauer ketone 24 (compound **2**) at the concentration found in fraction 5 was capable of significantly inhibiting IL-1 β and IL-6 secretion.

The potent anti-inflammatory activity found in *E. paradoxa* var. *paradoxa* suggests that it may be promising for medicinal use. If so,

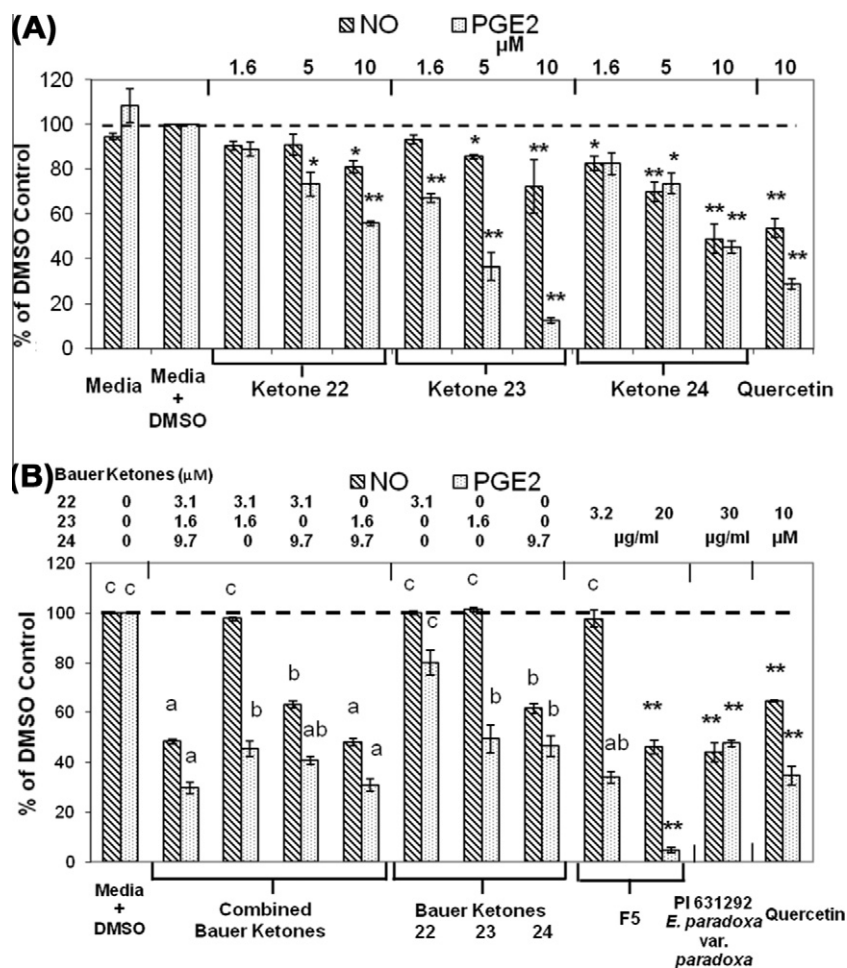


Fig. 5. Inhibition of NO and PGE2 production by combined or individual synthetic Bauer ketones in LPS-stimulated RAW264.7 cells. (A) Dose-dependent inhibition of NO and PGE2 production by synthetic Bauer ketones 22, 23 and 24 (compounds **10**, **1**, **2**). (B) NO and PGE2 levels after treatment with combined or individual synthetic Bauer ketones at the concentrations present in fraction 5 (Table 6). The highest and lowest doses were set at the concentrations of ketone 24 (compound **2**, 9.7 μM, rounded to 10 μM) and 23 (compound **1**, 1.6 μM) present in 3.2 μg/ml of fraction 5. Data were expressed as % of the (Media + DMSO + LPS) control ± SE (N = 3), and the (Media + DMSO + LPS) control group was normalized at 100% of NO production (11.4 ± 0.8 μM in (A), 23.2 ± 4.6 μM in (B)) and PGE2 production (2.6 ± 0.2 ng/ml in (A), 2.2 ± 0.3 ng/ml in (B)). Asterisks reflect significant differences (*p < 0.05 and **p < 0.001) from the (Media + DMSO + LPS) treatment group. Values labeled with different letters demonstrate a significant difference (p < 0.05) between them. Values with both a and b have significant difference from values with c only. Treatments without LPS revealed no change compared to (Media + DMSO) controls (data not shown).

its cultivation should be encouraged, as its native populations are limited and could be threatened by wild harvesting (Kindscher, 2006). Furthermore, identification of the key contributors, Bauer ketones 23 and 24 (compounds **1**, **2**), from *E. paradoxa* var. *paradoxa* by bioactivity-guided fractionation suggests their potential as anti-inflammatory agents *per se*. It is hoped that these findings on the identification of active constituents in *Echinacea* species may help guide the development of *Echinacea* preparations with improved efficacy as botanical dietary supplements.

4. Experimental

4.1. General experimental procedures

A semi-preparative HPLC system was used for fractionation, which consisted of a model 508 autosampler, a YMC-pack ODS-AM 250 × 10 mm C₁₈ column (YMC, Kyoto, Japan), a Beckman-Coulter System Gold with a 126 solvent module and a model 168 detector (Beckman Coulter, Fullerton, CA). GC-MS analysis was performed by using an Agilent Technologies gas chromatograph (6890 series), equipped with a capillary column (HP-5MS fused silica column coated with 5% diphenyl 95% dimethyl polysiloxane,

30 m, 250 μm bore, 0.25 μm film thickness) and a model 5973 mass spectrometer as detector (all from Agilent Technologies, Palo Alto, CA). DMEM, fetal bovine serum, sodium bicarbonate and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). DMSO, quercetin (compound **3**) and ursolic acid were purchased from Sigma-Aldrich (St. Louis, MO). CellTiter 96[®] Aqueous One solution Reagent and Griess reaction kit were from Promega (Madison, WI). Biotrek PGE2 enzyme immunoassay system (GE Healthcare, Piscataway, NJ) and BD OptEIA™ ELISA sets (BD Biosciences, San Diego, CA) were used for bioactivity assays. BCA Protein Assay Reagent was purchased from Pierce (Rockford, IL) and antibodies used in the Western blot were from Santa Cruz Biotechnology (Santa Cruz, CA).

4.2. Plant materials

Plant materials were obtained from the U.S. Department of Agriculture North Central Regional Plant Introduction Station (NCRPIS), Ames, IA. Accessions of *Echinacea* samples used in this study (Table 1) included: *E. angustifolia* (Ames 24996), *E. pallida* (Ames 28968), *E. pallida* (PI 631274), *E. paradoxa* (Ames 27724), *E. paradoxa* var. *paradoxa* (PI 631292) and *E. purpurea* (Ames

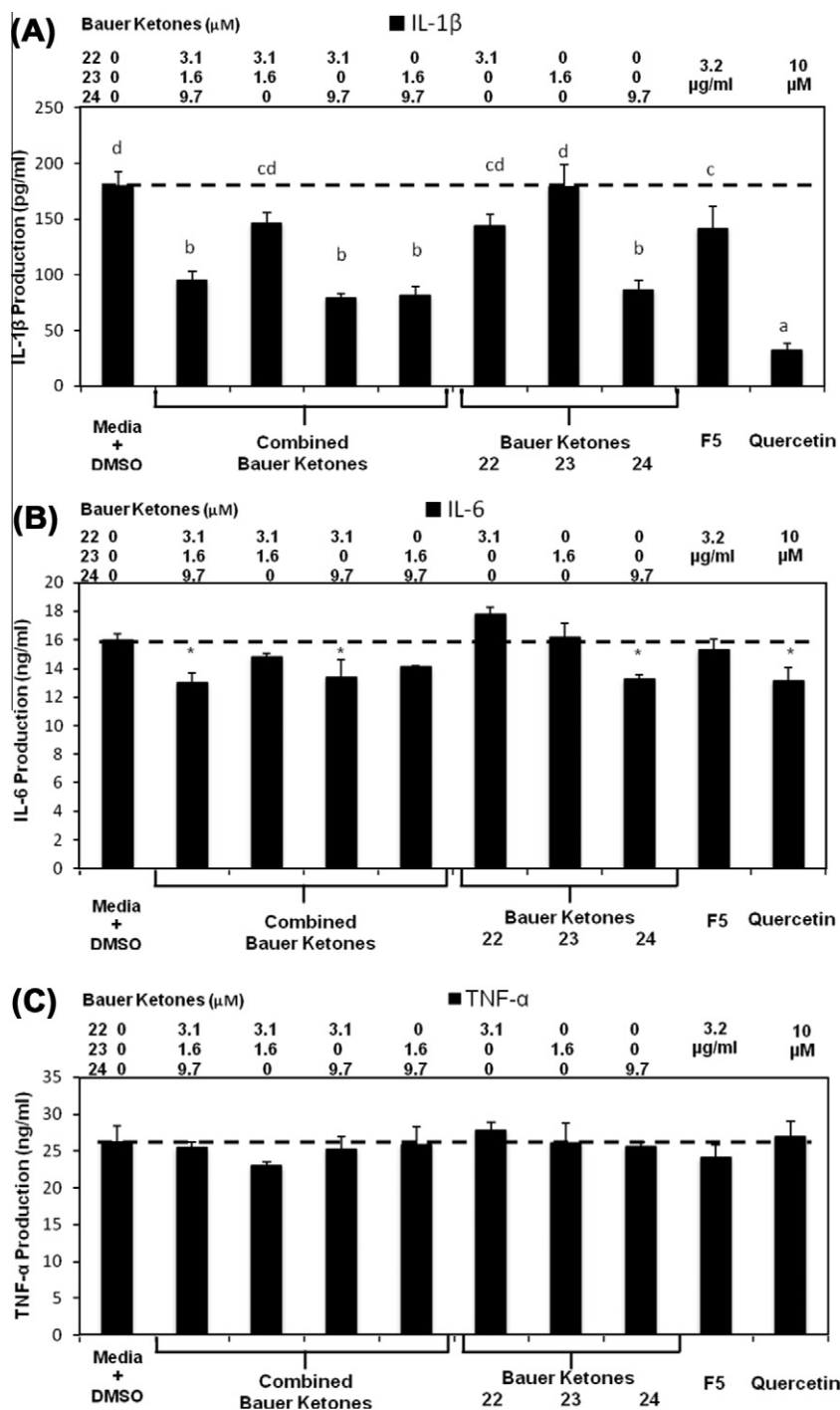


Fig. 6. Effects of combined or individual Bauer ketones at the concentrations present in fraction 5 (Table 6) on inflammatory cytokine production in LPS-stimulated RAW264.7 cells. The levels of IL-1 β (A), IL-6 (B) and TNF- α (C) after treatment with combined or individual Bauer ketones were compared to those of the (Media + DMSO + LPS) control group in 1 μ g/ml LPS induced RAW264.7 cells. Data were expressed as mean \pm SE (N = 3). Asterisks reflect significant differences (* p < 0.05 and ** p < 0.001) from the (Media + DMSO + LPS) control group. Values labeled with different letters demonstrate a significant difference (p < 0.05) between them. Values with two letters, i.e. cd, are not significantly different from values with either of these letters. Treatments without LPS revealed no change compared to (Media + DMSO) controls (data not shown).

27468). More detailed information about the original sources and availability of these accessions can be found in the Germplasm Resources Information Network (GRIN) database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Roots of these plants, harvested at the NCRPIS in November 2009, were dried for 8 days at 40 °C with constant humidity in a forced-air dryer. The dried materials were ground with a 40-mesh screen in a Wiley mill and then stored at –20 °C under N₂ prior to extraction.

4.3. Extraction

For each *Echinacea* accession, dried root powder (1 g) was extracted with EtOH–H₂O (95:5, v/v) for 6 h by using a Soxhlet extractor. After extraction, the ethanol solvent was removed with a rotary evaporator at 35 °C, followed by lyophilization, yielding the dried samples. The percentage yield of each accession (% of dried root powder) was as follows: Ames 24996 30.5%, Ames

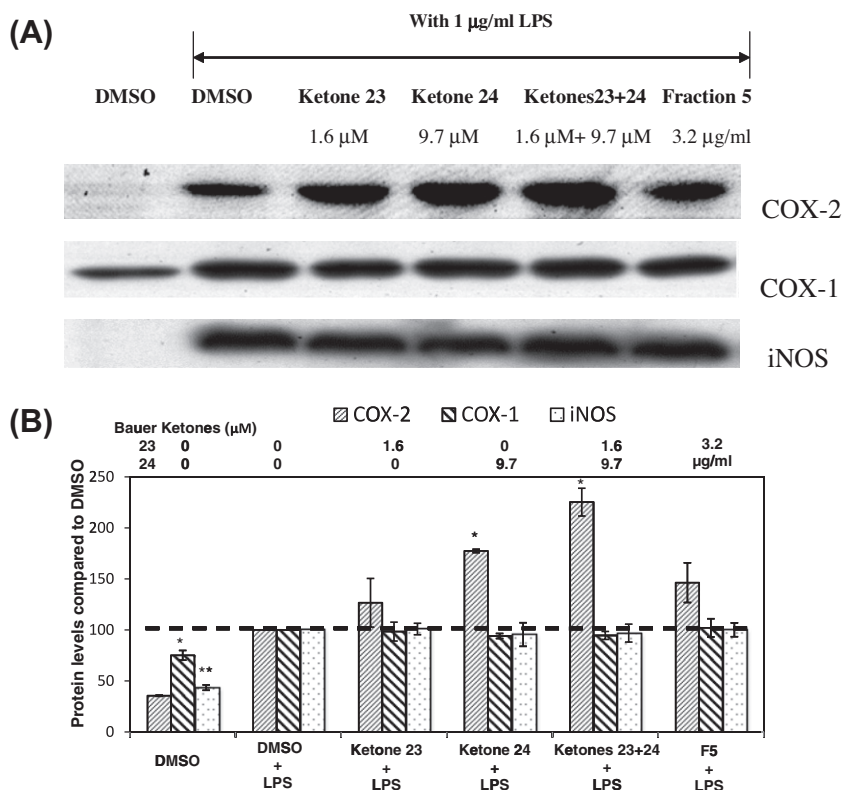


Fig. 7. Effects of combined or individual Bauer ketones on LPS-induced COX-2, COX-1 and iNOS protein levels in RAW264.7 cells. Treatments of controls, Bauer ketones or fraction 5 were applied to RAW264.7 cells with LPS induction. (Media + DMSO) without LPS addition was used as a negative control. (A) One replicate of protein (COX-2, COX-1 or iNOS) bands identified by Western blot. Bands were quantified with a densitometer, and results were shown in (B) as percentage of the (Media + DMSO + LPS) control \pm SE ($N = 3$ or 4). Asterisks reflect significant differences (* $p < 0.05$ and ** $p < 0.001$) from the (Media + DMSO + LPS) control group. α -Tubulin, as a loading control, was constitutively expressed among all treatments (data not shown).

28968 22.9%, PI 631274 16.9%, Ames 27724 20.5%, PI 631292 19.2% and Ames 27468 11.5%. The dried samples were redissolved in the minimal volume of DMSO that ensured complete sample solubilization. The concentrations of the ethanol extracts obtained from each of these accessions were 116, 78, 48, 66, 59 and 21 mg/ml, respectively. None of these extracts showed cytotoxicity in the cell viability assay (described as below), which was consistent with previous work by LaLone et al. (2007). The ethanol extracts of *Echinacea* species were stored at -20°C under argon. All extracts were assayed for endotoxin, and levels were undetectable by Lymulus Amebocyte Lysate assay, whose detectable level was above 0.0001 EU/ml (Bio Whittaker, Walkersville, MD).

4.4. Semi-preparative HPLC fractionation

Dried ethanol extract (2 g) of *E. paradoxa* var. *paradoxa* (PI 631292) was dissolved in EtOH–H₂O (42:58, v/v) and then filtered through a 0.45 μm filter. The filtrate was fractionated by using the semi-preparative HPLC system described in Section 4.1. Fractionation was conducted by using a mobile phase of 0.1% AcOH in endotoxin-free H₂O (solvent A) and CH₃CN (solvent B). The gradient of solvents A and B throughout the fractionation and the elution time periods for collecting each fraction are shown in Table 2. Fractions dissolved in DMSO were stored at -80°C under Ar to avoid degradation of potentially unstable Bauer ketones. In total, 6 fractions were obtained from the *E. paradoxa* var. *paradoxa* ethanol extract, with yields and original concentrations (mg/ml) shown in Table 3.

4.5. GC–MS analysis

The carrier gas for GC–MS analysis was He, at a flow rate of 1.2 ml/min. Oven temperature was initially 80°C for 2 min, then was increased to 200°C at a rate of $10^{\circ}\text{C}/\text{min}$ and held at 200°C for 5 min. Injector and detector temperatures were set at 250°C . The fractions in DMSO were dried with nitrogen gas and then redissolved in CH₃CN. One microliter of *Echinacea* fraction or synthesized standards was injected automatically in splitless mode. Compounds in each fraction were identified by comparing their retention times and mass spectral data with those in the NIST Mass Spectral Library (NIST 08). Pentadeca-8Z-ene-11, 13-diyn-2-one (Bauer ketone 23, compound 1) and pentadeca-8Z, 13Z-dien-11-yn-2-one (Bauer ketone 24, compound 2) were quantified in our fractions on the basis of synthesized Bauer ketones as internal standards. The amount of added standard was adjusted for the small amount of impurities present in the standard preparations. Tetradeca-8Z-ene-11, 13-diyn-2-one (Bauer ketone 22, compound 10) was quantified in relation to Bauer ketones 23 and 24. The standard error for the measurement of all Bauer ketones was 3.22%, based on three measurements of synthesized Bauer ketones.

4.6. Bauer ketone synthesis

Bauer ketones were chemically synthesized following the procedure developed by Kraus et al. (2007) and described in detail in the Ph.D. thesis of Jaehoon Bae (Bae, 2006). Based on ^1H - and ^{13}C -NMR spectra (Kraus et al., 2007), the percent purity for synthetic Bauer ketones 22, 23 and 24 (compounds 10, 1, 2) was

~80%, 90% and 99%, respectively. All synthetic Bauer ketones were stored at -80°C under Ar to avoid degradation.

4.7. Cell culture and treatments

RAW264.7 mouse macrophage cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as previously described (Hammer et al., 2007) except for an adjustment to our incubator to hold cells at 95% relative humidity. The medium for culturing RAW264.7 cells was high glucose DMEM supplemented with 10% FBS, 2% sodium bicarbonate and 1% penicillin/streptomycin.

Prior to treatment, 0.64×10^5 cells/well (for NO and cytokine) or 1×10^5 cells/well (for PGE2) were plated in 24-well tissue-culture plates with 0.5 ml culture medium/well and incubated for 24 h. DMSO vehicle control, a quercetin (compound **3**) positive control, and *Echinacea* extracts, fractions or synthesized compounds were diluted into cell media for a final DMSO concentration of 0.1% v/v, well below the toxic level of DMSO. Diluted samples were applied to cells with/without 1 $\mu\text{g}/\text{ml}$ LPS simultaneously. Following treatment for 8 h (for PGE2) or 24 h (for NO and cytokines), supernatants were collected for storage at -80°C (for PGE2 and cytokines) or for immediate NO measurement. For cell viability assays, 1×10^4 cells/well were plated in 48-well tissue-culture plates with 0.2 ml culture medium/well and incubated for 24 h. Diluted samples including (Media + DMSO) control, *Echinacea* extracts, fractions, synthesized compounds and ursolic acid controls in cell media were applied to cells without LPS stimulation for 24 h. For all experiments, three individual plates from three corresponding flasks of cells were included as three replicates.

4.8. Cell viability assay

Following 24 h treatment, supernatants were replaced by 200 μl fresh media and 30 μl of CellTiter 96[®] AQueous One solution Reagent for each well. After incubation for 195 min, 200 μl of supernatant containing dye product was transferred from each well to 96-well plates, followed by reading absorbance at 562 nm. There were three repeated treatments within each plate. Viability compared to (Media + DMSO) control was denoted as the mean absorbance of *Echinacea* extracts, fractions or synthesized compound divided by the mean absorbance of (Media + DMSO) control, expressed as a percentage.

4.9. NO, PGE2 and cytokine measurement

After the supernatants were collected, Griess reaction was used to determine NO levels according to procedures as described (Huang et al., 2009). For PGE2 measurement, samples stored at -80°C were thawed at room temperature and then diluted 15-fold into ddH₂O. Biotrek PGE2 enzyme immunoassay system was employed to measure PGE2 concentrations in cell supernatants based on manufacturer's protocols. The levels of TNF- α , IL-1 β and IL-6 in the supernatant samples were analyzed with BD OptEIA[™] ELISA sets according to manufacturer's instructions. Before the procedures were performed, the samples were diluted 20- to 40-fold into assay diluent for TNF- α and IL-6 to ensure that sample concentrations were within the linear range of a standard curve. Concentrations of NO, PGE2 and each type of cytokine produced by our samples were interpolated from their corresponding standard curves.

4.10. Western blot analysis

RAW264.7 cells at 80% confluency in 10 cm Petri dishes were treated with DMSO, *Echinacea* extracts and 100 μM quercetin

(compound **3**) plus 1 $\mu\text{g}/\text{ml}$ LPS and DMSO alone for 8 h (for COX2 and COX1) or for 24 h (for iNOS). Preparation of whole-cell protein extracts from treated cells was performed as described by LaLone et al. (2010). The protein concentration was measured by using the BCA Protein Assay Reagent. An equal amount of total protein for all treatments was loaded to detect iNOS, COX2, COX1 and α -Tubulin. The iNOS (sc-7271), COX-1 (sc-19998), COX-2 (sc-19999) and α -Tubulin (sc-8035) mouse monoclonal antibodies were diluted 1:600, 1:800, 1:500 and 1:1000, respectively, in 7% milk Tris-buffered saline containing 0.1% Tween 20. The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005), was diluted 1:1000 in the above-mentioned solution. The immunoblot was carried out as described (Przybylski et al., 2001). The signals were detected by ECL and quantified by Quantity One Software as described by Hammer et al. (2007).

4.11. Statistical analysis

A randomized complete block design was applied to all experiments. For each experiment, there were three plates of cells receiving the same treatments. The Media + DMSO \pm LPS treatment within each plate was normalized to 100% and three plates were considered as fixed blocks. Because of the normalization the control values appear in the results without standard error (SE). For the cytotoxicity assay, there were three repeated measurements in each plate, which were averaged. For PGE2 assays and ELISA, concentrations of PGE2 and cytokines for all treatments were log transformed. Results were presented as% of Media + DMSO \pm LPS \pm SE. ANOVA analysis using GLM procedures in SAS 9.0 (SAS Institute Inc., Cary, NC) was conducted to test statistical differences as compared to the Media + DMSO \pm LPS controls. For combined Bauer ketone treatments, multiple comparison with Tukey adjustment was conducted to test statistical differences among them.

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